ABR1, an APETALA2-Domain Transcription Factor That Functions as a Repressor of ABA Response in Arabidopsis[1]

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The plant hormone abscisic acid (ABA) plays an important role in plant development and stress responses. An important step of ABA action is activation or inactivation of gene expression. Although several transcription factors are identified to function as positive regulators of ABA-induced gene expression, little is known about the negative regulators of ABA-regulated gene expression. Here, we have identified an APETALA2 (AP2) domain transcription factor that serves as a repressor of ABA response during seed germination and ABA- and stress-induced gene expression in Arabidopsis (*Arabidopsis thaliana*). The expression of the AP2-like ABA repressor 1 (*ABR1*) gene itself was responsive to ABA and stress conditions including cold, high salt, and drought. Disruption of *ABR1* led to hypersensitive response to ABA in seed germination and root growth assays. The mutant plants were also hypersensitive to osmotic stress conditions, such as high salt and high concentrations of mannitol. Further analyses indicated that increased stress sensitivity may result from hypersensitivity to ABA as ABA biosynthesis inhibitor rescued the stress hypersensitivity phenotype. The *abr1* mutant plants accumulated significantly higher levels of ABA- and stress-inducible gene transcripts as compared to the wild-type plants, supporting the hypothesis that this AP2 domain protein serves as a repressor of ABA-regulated gene expression.

The plant hormone abscisic acid (ABA) regulates many agronomically important aspects of plant development and physiology, including seed maturation and dormancy, as well as responses to environmental stress conditions, such as drought, salinity, and low temperature (Fedoroff, 2002; Finkelstein et al., 2002; Himmelbach et al., 2003). Gene regulation and inhibition of seed germination provide useful bioassays for both forward and reverse genetic analyses that have revealed several molecular components in plant ABA signal transduction pathways (Giraudat, 1995; Finkelstein et al., 2002). These components range from early signaling intermediates, such as G proteins and protein kinases/phosphatases, to transcription factors (TFs) and RNA metabolic proteins implicating a complex molecular network in the modulation of ABA responses in plants.

A critical model system for studying ABA response in plants is ABA-regulated gene expression. Characterization of gene promoters indicates that many gene promoters contain cis-acting elements that are responsible for ABA regulation. These elements are often referred to as ABA-responsive elements (ABRE; Guiltinan et al., 1990). Transacting factors interacting with the ABRE have been recently identified and shown to function in ABA response (Foley et al., 1993; Choi et al., 2000; Uno et al., 2000; Kang et al., 2002; Kizis and Pages, 2002; Kim et al., 2004). Among the components identified by seed germination genetic screens, several ABA INSENSITIVE (ABI) loci have been shown to encode TFs, including ABI3, ABI4, and ABI5 (Giraudat, 1995; Finkelstein et al., 2002). These TFs are expressed in the developing seeds, mature seeds, and young seedlings (Finkelstein et al., 2002). As a result, ABI3 to ABI5 function in the ABA-mediated regulation of seed development, germination, and early growth of seedlings (Finkelstein et al., 2002; Arroyo et al., 2003). The *ABI3*, *ABI4*, and *ABI5* genes encode TFs of the B3 domain, the APETALA2 (AP2) domain, and bZIP factor class, respectively (Finkelstein et al., 2002). Mutations in these genes resulted in ABA-insensitive seed germination, while overexpression of these genes leads to ABA hypersensitivity (Koornneef et al., 1984; Giraudat et al., 1992; Finkelstein, 1994; Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001; Finkelstein et al., 2002). These findings indicate that ABI3, ABI4, and ABI5 are both required and sufficient for ABA signaling, although they are synergistic to each other and act as positive regulators of ABA response in Arabidopsis (*Arabidopsis thaliana*). Some negative regulators of ABA responses have also been identified by screening for ABA hypersensitive mutants. These include ABA signal regulators, such as ROP10 (the Rho-like small G protein); ERA1 (farnesyltranferase β-subunit; for review, see Himmelbach et al., 2003); CBL9, a calcium sensor; calcineurin B-like 9 (Pandey et al., 2004); and

[1] This work was supported by grants from the U.S. Department of Agriculture and the National Science Foundation (to S.L.).

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.105.066324.

CIPK3, a calcineurin B-like-interacting protein kinase 3 (Kim et al., 2003). Another category of negative regulators are RNA metabolism proteins including HYL1, a double-stranded RNA-binding protein; ABH1, an mRNA cap-binding protein; and SAD1, an SM-like snRNP protein S (for review, see Himmelbach et al., 2003).

The AP2 domain TFs were named after the original gene, *AP2*, shown to function in flower organ development (Jofuku et al., 1994; Riechmann and Meyerowitz, 1998). More recently, AP2 protein was shown to play a role in regulation of seed size and yield (Jofuku et al., 2005; Ohto et al., 2005). Recent genomic analyses provided an overview of the AP2-domain proteins, a large diverse family of plant-specific TFs (Sakuma et al., 2002; Gong et al., 2004; Gutterson and Reuber, 2004). This protein family comprises approximately 145 gene products and is classified into five subfamilies based on similarities in their DNA-binding domains: AP2 subfamily (14 genes), RAV subfamily (six genes), DREB subfamily (55 genes; group A), ERF subfamily (65 genes; group B), and others (four genes). A number of AP2-domain proteins were identified as TFs that control the expression of various genes, including abiotic stress-responsive genes, ethylene-responsive genes involved in ethylene, salicylic acid, and jasmonic acid responses and disease resistance (Sakuma et al., 2002; Gutterson and Reuber, 2004). All the AP2 proteins that are responsive to ethylene were referred to as ERF (Sakuma et al., 2002; Gutterson and Reuber, 2004). The AP2-domain proteins for abiotic stress-responsive gene expression were referred to as DREBs and CBFs defined in the course of analyzing drought- and cold-responsive gene expression (Thomashow, 1999, 2001; Shinozaki and Yamaguchi-Shinozaki, 2000; Kizis et al., 2001). During the study of ABA-regulated gene expression, we have uncovered an AP2-domain protein, ABA repressor 1 (ABR1), which is strongly responsive to ABA and functions as a negative regulator of ABA responses.

RESULTS

The *ABR1* Gene Is Highly Induced by ABA and Stress in Arabidopsis

In our earlier studies, we have identified calcineurin B-like protein CBL9 and its interacting kinase (CIPK3) as regulators of ABA responses (Kim et al., 2003; Pandey et al., 2004). To identify the downstream target genes of CIPK3, we performed microarray analysis of *cipk3* mutant and wild-type plants under ABA treatment. A number of genes were found to be differentially induced by ABA in the *cipk3* mutant plants. In particular, expression of the *ABR1* gene (At5g64750), annotated to be an AP2-domain TF, was highly induced by ABA, and this induction level was reduced 2.6-fold after 2 h of ABA treatment in the *cipk3* mutant (J. Grant and S. Luan, unpublished data). We suspected that the *ABR1* gene may be regulated by the CBL9-CIPK3-mediated ABA-signaling pathway and decided to study its possible function in ABA response.

The *ABR1* gene encodes an ERF member (B subfamily), B-4 of ERF/AP2 TF (Sakuma et al., 2002; Gutterson and Reuber, 2004). There are seven members in the ERF subfamily and all of them contain one AP2 domain. As a step toward functional analysis, we

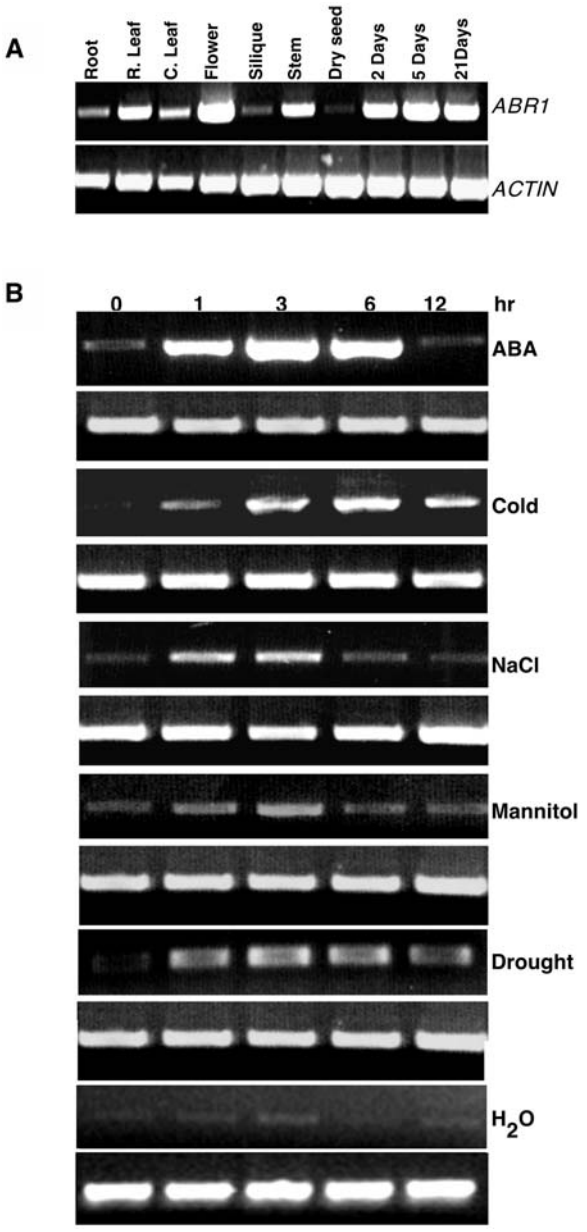

Figure 1. Expression pattern of the *ABR1* gene. A, RT-PCR analysis of *ABR1* transcripts during seed germination and in different organs of Arabidopsis plants. Total RNA was isolated from various tissues and stages (root, rosette leaf, cauline leaf, flower, silique, stem, and dry seeds) of wild-type plants grown under long-day conditions or from germinating seeds and young seedlings (2, 5, and 21 d after sowing). Thirty-five cycles of RT-PCR were performed with *ABR1*-specific primers (top gel) or *Actin2*-specific primers (bottom gel). B, Four-day-old seedlings grown on MS medium were treated with ABA (100 μM), NaCl (300 mM), cold (4°C), mannitol (400 mM), and drought (dehydration), and 25 cycles of RT-PCR analyses performed with *ABR1* gene-specific primers. *Actin2*-specific primers were used for control RT-PCR analyses.

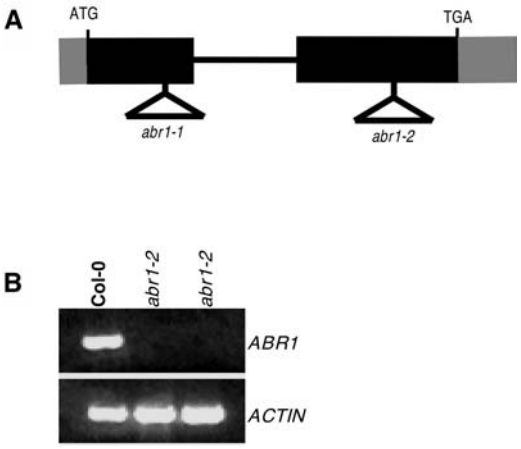

Figure 2. Isolation of the *abr1* T-DNA insertional mutants. A, Intron-exon organization of the Arabidopsis *ABR1* gene (coding region) and T-DNA locations. Gray shaded boxes indicate untranslated regions, and solid black boxes and line indicate coding regions and intron, respectively. The position of the T-DNA insertion in two alleles, *abr-1* and *abr1-2*, are indicated by triangles (not to scale). B, RT-PCR analysis of *ABR1* transcripts in wild-type (Col-0) and mutant (*abr1-1* and *abr1-2*) alleles. Expression of *Actin2* was analyzed as a loading control.

examined the expression pattern of this gene in Arabidopsis plants using a reverse transcription (RT)-PCR procedure. These analyses revealed that the *ABR1* gene is expressed ubiquitously in all developmental stages of plants and in all organs examined, including root, leaf, stem, flower, and silique, although expression levels in silique and seeds are rather low (Fig. 1A). In addition to induction by ABA, the *ABR1* gene is highly induced by several stress conditions, including salt (300 mM NaCl), drought (dehydration), and cold (4°C; Fig. 1B). A rapid accumulation of *ABR1* transcript was observed at 1 h after ABA treatment and the strongest accumulation was observed at 3 h, followed by a decrease of transcripts to the basal level at 12 h of treatment. Under cold stress treatment, *ABR1* transcripts accumulated to a maximal level at 6 h followed by a reduction at 12 h. Similarly, a transient accumulation of transcripts was observed at 3 h for both high salt and drought treatment. The induction by ABA and each stress followed slightly different kinetic patterns. Induction by ABA was more rapid and more robust as compared to the induction by NaCl, cold, and drought.

The *abr1* Mutant Is Hypersensitive to ABA, Osmotic Stress, and Glucose

The ABA- and stress-inducible expression suggested a possible role of the *ABR1* gene in ABA-mediated and stress signal transduction pathways. To examine the function of the *ABR1* gene, we isolated two T-DNA insertional alleles of this gene (*abr1-1* and *abr1-2*) from the collections of T-DNA-transformed Arabidopsis lines (SAIL140_G06 and SALK_012151, Arabidopsis Biological Resource Center). Homozygous mutant lines were established after selfing. Sequence analysis indicated that the insertional site in the *abr1-1* allele is located in the first exon at 488 bp after ATG and the T-DNA is inserted in the second exon following the 1,640th bp after ATG in the *abr1-2* allele (Fig. 2A). The RT-PCR analysis showed that both insertions abolished the expression of *ABR1* (Fig. 2B). As a control, we noted that expression of the *Actin2* gene was not affected in the *abr1* mutants (Fig. 2B).

To evaluate the consequences of *ABR1* gene disruption, we examined the mutant plants under normal growth conditions and found them to be indistinguishable from the wild type (data not shown). As the *ABR1* gene is regulated by ABA and abiotic stress signals, we speculated that *ABR1* may function in plant responses to stress and ABA. We tested this hypothesis by several assays, such as seed germination, adult plant stress tolerance, and stomatal closure

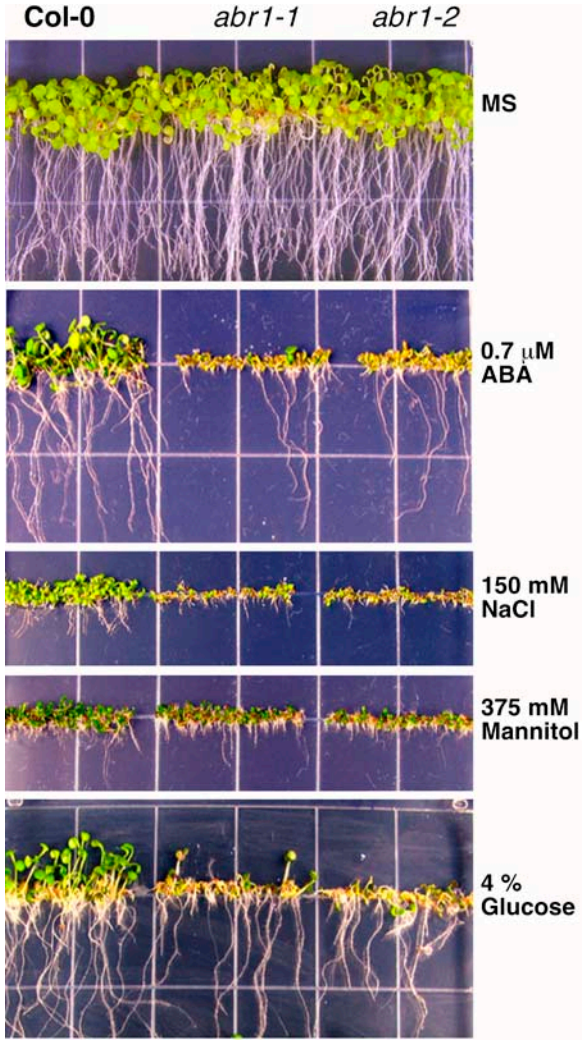

Figure 3. Germination of *abr1* mutant seeds is hypersensitive to ABA, Glc, and osmotic stress conditions. Wild-type (Col-0), *abr1-1*, and *abr1-2* mutant seeds on MS agar medium or MS agar medium containing 0.7 μM ABA, 125 mM NaCl, 375 mM mannitol, or 4% Glc were incubated at 4°C for 6 d before transfer to 23°C for germination. The photograph was taken on day 12 after transfer to 23°C.

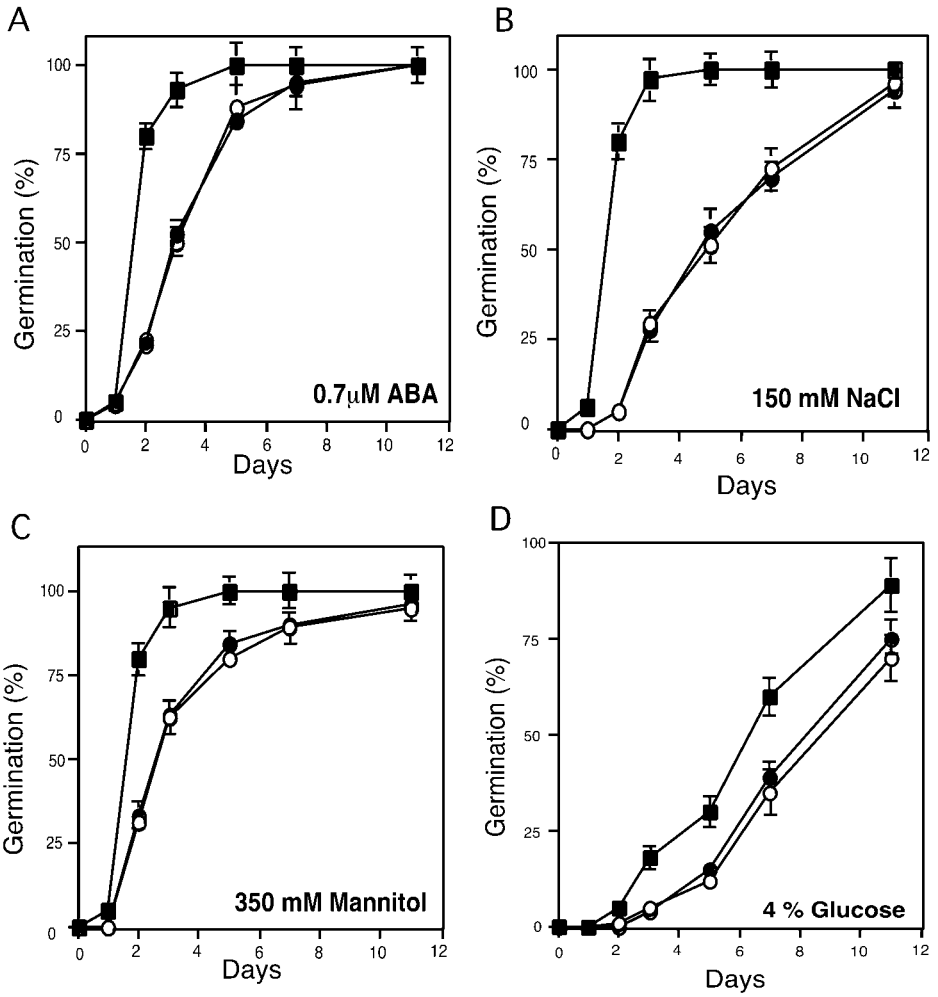

Figure 4. Germination time courses (days after incubation at 23°C) on medium containing 0.7 μM ABA, 150 mM NaCl, 350 mM mannitol, or 4% Glc. Wild-type (Col-0) and *abr1-1* and *abr1-2* mutant alleles are represented as black squares, black circles, and white circles, respectively. Results in A to D are presented as average values with standard errors from three experiments.

assays under appropriate abiotic stress conditions. In the germination assays, the mutant seeds exhibited hypersensitivity to ABA as compared to the wild-type seeds (Fig. 3). In addition, mutant seeds and seedlings were hypersensitive to osmotic stress (mannitol), high salt, and Glc. As shown in Figure 3, the germination and subsequent growth of mutant seedlings were comparable to the wild type on the normal medium (Murashige and Skoog [MS]), but were significantly more inhibited by ABA, Glc, and osmotic stress. More detailed analyses of germination rates under different concentrations of ABA and various stress conditions were performed to evaluate the *abr1* mutant. Germination in the wild type was not affected by ABA concentration less than 0.5 μM, but germination of mutant seeds was significantly inhibited at 0.3 μM ABA (data not shown). At 0.7 μM ABA, more than 75% of wild-type seeds germinated, whereas only 20% of mutant seeds germinated in the 2 d after transferring to 23°C (Fig. 4A). We noted that ABA sensitivity of the *abr1* mutant in germination was comparable to some previously reported mutants, such as *cbl9* and *cipk3* (Kim et al., 2003; Pandey et al., 2004), but was lower than some other mutants, such as *era1*, *abh1*, and *hyl1* (Cutler et al., 1996; Lu and Fedoroff, 2000; Hugouvieux et al., 2001). At 150 mM NaCl, 80% of wild-type seeds germinated within 2 d, but the germination rate for the mutant seeds was less than 5% (Fig. 4B). Similarly, germination rate of wild-type and mutant seeds was 77% and 30%, respectively, on the medium containing 350 mM mannitol (Fig. 4C). The germination rate of the *abr1* mutant and the wild type in 4% Glc medium was also significantly different (4% versus 20%; Fig. 4D). These analyses with both mutant alleles (Fig. 4) supported the conclusion that mutation of the *ABR1* gene renders the seedlings hypersensitive to ABA, osmotic stress, high salt, and Glc.

As hyperosmotic stress and high salt induce production of ABA in plants (Leung and Giraudat, 1998; Seo and Koshiba, 2002), hypersensitivity of the *abr1* mutant to these stress conditions could result from increased sensitivity to ABA or from increased production of ABA, or both. In other words, altered stress sensitivity may be an ABA-dependent or -independent process. To distinguish these possibilities, we included an inhibitor for ABA biosynthesis, norflurazon (NF), in the germination medium as previously described (Zeevaart and Creelman, 1988; Kim et al., 2003; Pandey

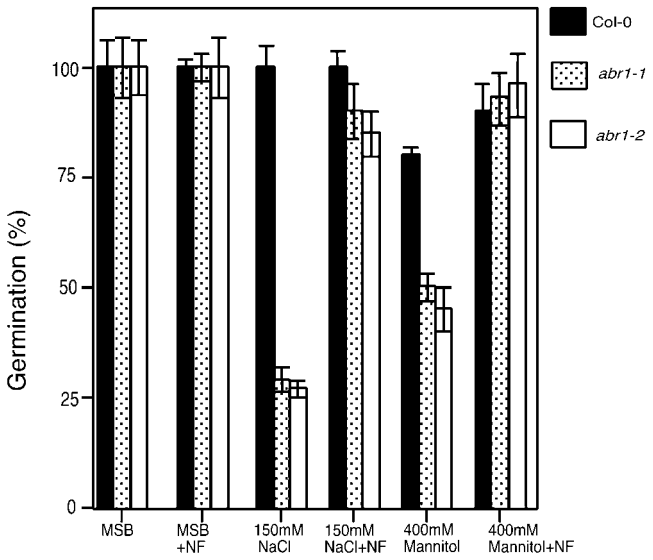

Figure 5. Effect of the ABA biosynthesis inhibitor NF on germination. Germination of wild-type and *abr1* mutant seeds on medium containing 150 mM NaCl or 400 mM mannitol with or without 100 μM NF. Seed germination rate was scored 3 d after transfer to a 23°C growth chamber.

et al., 2004). Under normal conditions, 100 μM NF did not affect the germination rate of wild-type or mutant seeds in a 3-d germination assay. On the stress media (containing 150 mM NaCl or 400 mM mannitol), 100% and 80% of wild-type seeds germinated in the 3-d incubation on NaCl and mannitol, respectively. In contrast, only 25% and 50% of the *abr1-1* and 22% and 45% of the *abr1-2* mutant seeds germinated on the NaCl- and mannitol-containing media. In the presence of 100 μM NF, however, the germination rate of the mutant seeds on NaCl and mannitol was restored to 90% and 95% for *abr1-1* and 85% and 97% for *abr1-2*, respectively. These results indicate that osmotic stress and salt might have exerted their inhibitory effect on mutant seed germination through an ABA-dependent process (Fig. 5).

In the course of the germination assays, we noted that the postgermination development of *abr1* mutant alleles appeared to be more sensitive to ABA and stress media as compared to the wild-type seedlings (Figs. 3 and 4). To further corroborate this observation, we germinated the wild-type and mutant seeds on normal medium and subsequently transferred comparable seedlings to ABA-containing or stress media after 3 d of growth. Root elongation was then scored and compared between the wild-type and mutant alleles. Because root growth is inhibited by higher levels of ABA as compared to those used in the germination assays (Gosti et al., 1999; Beaudoin et al., 2000), we used different concentrations of ABA for the root growth assays. Figure 6 showed that root growth of both *abr1* mutant alleles was significantly more inhibited than the wild-type seedlings. However, we did not observe a significant difference in root growth between wild-type and mutant seedlings on Glc or osmotic stress medium (data not shown). The results here suggest that *ABR1* not only regulates ABA responses at the germination stage, but also affects postgermination developmental processes mediated by ABA.

Disruption of *ABR1* Enhanced ABA- and NaCl-Induced Gene Expression

We have shown that the *ABR1* gene is activated strongly by stress conditions and ABA. Furthermore, germination assays revealed an increased sensitivity of the *abr1* mutant under ABA exposure and osmotic stress conditions. All these results implicate ABR1 in the regulation of stress and ABA responses in plants. Because *ABR1* is a member of the TF family, we speculated that it may play a role in gene regulation. Several ABA- and stress-induced genes were used as markers to test this hypothesis. These gene markers included *RD29A*, *RD29B*, *RD22*, *RAB18*, *COR47*, and *COR15A* that have been previously used for monitoring the ABA and stress response pathways in plants (Gilmour et al., 1992; Kurkela and Borg-Franck, 1992; Lang and Palva, 1992; Lin and Thomashow, 1992; Yamaguchi-Shinozaki and Shinozaki, 1994; Tahtiharju et al., 1997; Liu et al., 1998; Shinozaki and Yamaguchi-Shinozaki, 2000; Cheong et al., 2003; Kim et al., 2003; Pandey et al., 2004). We compared the *abr1* mutant and the wild-type plants in the expression patterns of these genes.

Upon ABA treatment, all gene markers were induced in both the wild-type and *abr1* mutant allele seedlings (Fig. 7). However, ABA-mediated induction of these genes in both *abr1* mutants was higher than the induction in wild-type plants. In particular, the induction of *RAB18*, *RD22*, *COR47*, and *COR15A* was

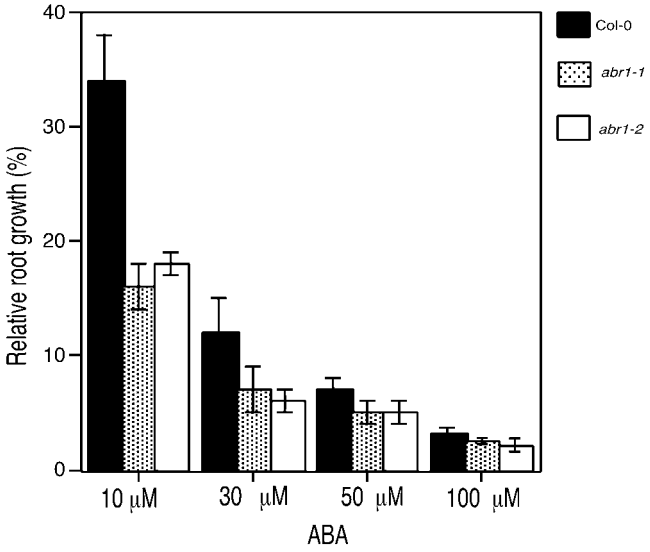

Figure 6. Postgermination root growth in *abr1* mutants was hypersensitive to ABA. Three-day-old seedlings of Col-0 and *abr1* mutant alleles were transferred from MS medium to MS medium supplemented with various concentrations of ABA. The root length was measured 2 weeks after the transfer. Results presented as average values with standard errors from three experiments.

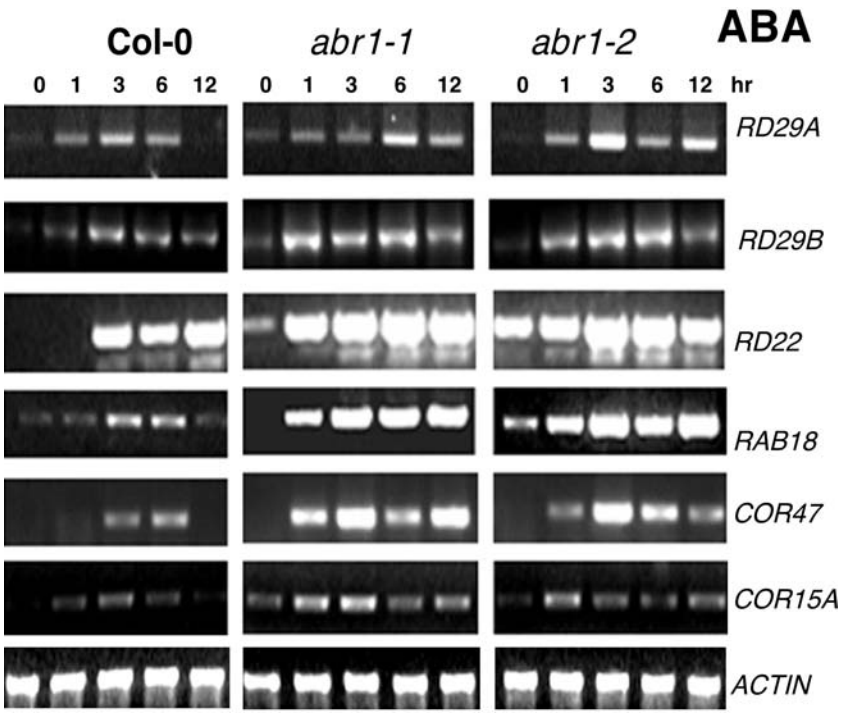

Figure 7. Expression of stress-responsive genes in wild-type (Col-0) and *abr1* mutants (*abr1-1* and *abr1-2*) after ABA treatment. Four-day-old seedlings grown on MS medium were treated with ABA (100 μM), and 25 cycles of semiquantitative RT-PCR analyses were performed with respective stress marker gene-specific primers. *Actin2*-specific primers were used in the RT-PCR analysis as an internal quantitative control.

much stronger in both *abr1* mutants than in wild type, although the extent and kinetics of induction were different among different markers. In the mutant plants, hyperinduction of marker genes, including *RD22*, *COR47*, and *RD29B*, was also apparent under high salt conditions (Fig. 8). Therefore, *ABR1* appears to act as a negative regulator in ABA- and NaCl-responsive gene expression, consistent with the results from seed germination assays.

In some cases, levels of stress gene expression correlate with stress tolerance in plants (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Cheong et al., 2003). But in other cases, such modification of stress gene expression appears to be insufficient to induce tolerance changes (Kim et al., 2003; Pandey et al., 2004). We assayed dehydration, salinity, and freezing stress tolerance of wild-type and *abr1* mutant plants and found no significant difference (data not shown).

DISCUSSION

The mechanisms underlying ABA responses in plants have been intensively studied by biochemical

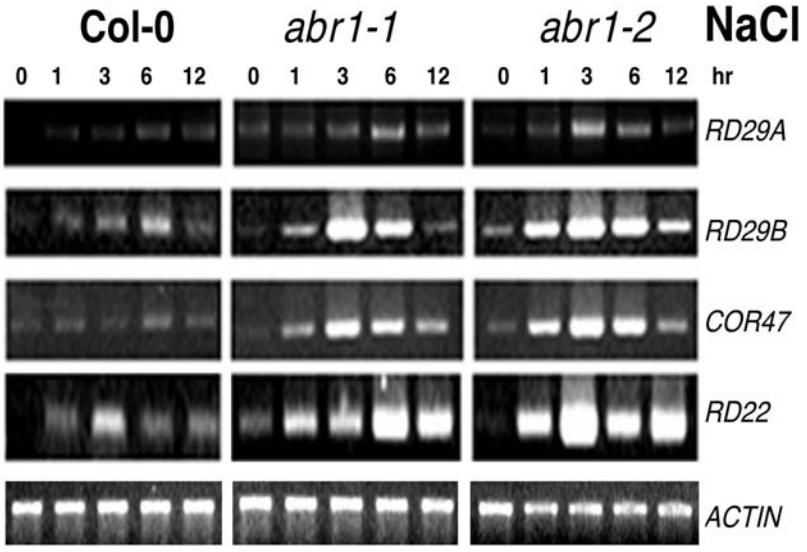

Figure 8. Expression of stress-responsive genes in wild-type (Col-0) and *abr1* (*abr1-1* and *abr1-2*) mutants after NaCl treatment. Semiquantitative RT-PCR analyses (25 cycles) with RNA isolated from NaCl-treated plants. *Actin2*-specific primers were used in the RT-PCR analysis as internal quantitative control. Experiments were repeated three times and results from one representative experiment are shown.

and genetic approaches. Studies have identified a number of components in the molecular network linking the ABA signal to the cellular responses in plant cells. Such components are broadly defined into two large categories: signal transducers and TFs. The signal transducers include protein kinases and phosphatases, G proteins (both trimeric and small GTP-binding proteins), RNA metabolic proteins, phospholipases, and so on (for review, see Rock, 2000; Finkelstein et al., 2002; Himmelbach et al., 2003). The TFs include ABA response element-binding proteins (AREBs/bZIPs), which include ABI5/AtDPBF1, AtDPBF2, AtDPBF3/AREB3, AtDPBF4, AtDPBF5/ABF3, ABF1, ABF2/AREB1,GBF3, DPBF1, -2, -3, TRAB1, PvZIP6, ROM2, EmBP-1, AtbZIP12, AtbZIP 13, AtbZIP 14, AtbZIP 15, AtbZIP 27, and AtbZIP 67; RY/Sph elements/B3 domain proteins (ABI3, AfVPI, CpVP1, C-ABI3, PvALF, PtABI3, OsVP1, TaVP1, and VP1); MYB (AtMYB2); MYC (AtMYC); HD-Zip (ATHB6, ATHB7, and ATH12); and AP2 (ABI4) (Bensmihen et al., 2002; Finkelstein et al., 2002). It is noteworthy that the signal transducers identified thus far can function to enhance or repress ABA responses, whereas the known TFs, with the exception of ROM2 and AtbZIP12/EEL, which act as repressors of PvALF and AtEm-1-activated transcription, respectively (Chern et al., 1996; Bensmihen et al., 2002), all play a positive role in ABA responses (required for maximal response). Our study here has identified an AP2-domain TF that fits the role of a negative regulator in the ABA responses.

Regarding ABA response, one TF, ABI4, has been shown to belong to the AP2 superfamily. This protein was identified in a genetic screen for ABA resistance (or insensitivity) during seed germination (Finkelstein et al., 1998). Later studies have shown that maize ZmABI4, a homolog of Arabidopsis ABI4, can bind to the *CE1* element in its own promoter and in the ABA- and sugar-responsive genes (Niu et al., 2002). In the context of gene regulation by ABA, several other ABIs (ABI3 and ABI5) are also TFs that interact and have a synergistic relationship in the activation of ABA-induced gene expression. The ABFs are ABI5-like TFs that belong to bZIP-type DNA-binding proteins (Choi et al., 2000). Interestingly, ABI5 and AtbZIP12/EEL, an ABI5-like protein, were shown to function antagonistically to fine tune the gene expression at late embryogenesis (Bensmihen et al., 2002).

Our work in this article provides evidence that an AP2-domain TF, unlike ABI4, which functions as a positive regulator, can serve as a negative regulator of ABA-induced gene expression. This finding is significant because it expands a new framework for the understanding of ABA-regulated gene expression. First, the finding of a putative TF in the negative regulation of ABA response provides a potential link to the signal transducers that have been shown to repress ABA responses (Hugouvieux et al., 2001; Lemichez et al., 2001; Kim et al., 2003; Lopez-Molina et al., 2003; Leonhardt et al., 2004; Pandey et al., 2004). Second, ABR1 may functionally interact with the ABI4 in the regulation of ABA-responsive genes. It is conceivable that ABR1 and ABI4 may bind to the similar cis-acting elements and antagonize each other in their action, as reported for the interaction between ABI5 and AtbZIP12/EEL (Bensmihen et al., 2002). In addition, negative regulation of ABA responses by AP2 TF clearly fortifies a common paradigm in gene regulation: Both positive and negative regulators coordinate to control the level of gene expression. In this article, several gene markers were used to study the function of ABR1 in ABA-responsive gene expression. Some of the gene markers, such as *RAB18*, are also regulated by ABI4 (Soderman et al., 2000). But the regulatory effect of ABI4 and ABR1 was exactly opposite: ABI4 is required for maximal expression and ABR1 represses this expression. Further work will test whether ABR1 indeed interacts directly with the coupling element, *CE1* (core consensus sequence, CACCG), cis-acting elements for ABI4 binding. Also genome-wide transcription profiling will help understand the ABR1 regulons and how these genes correlate with the promoter elements present in the ABR1- and ABI4-regulated genes.

It is noteworthy that *ABR1* was identified in a study intended to isolate target genes for a calcium-regulated protein kinase, CIPK3. The CIPKs are protein kinases that are regulated by the calcineurin B-like calcium sensors called CBLs (for review, see Luan et al., 2002; Kolukisaoglu et al., 2004). As CIPK3 appears to function as a negative regulator of ABA response in Arabidopsis (Kim et al., 2003), we reasoned that it must regulate ABA-responsive genes. A microarray study using the whole-genome array did recover a number of genes that are regulated differently in the *cipk3* mutant as compared to the wild type upon ABA treatment. One of these genes is *ABR1*, which is very strongly regulated by ABA. Because CIPK3 serves as a negative regulator of ABA response and *ABR1* gene expression was reduced in the *cipk3* mutant, we speculated that this *ABR1* could function in a similar manner as CIPK3 and may serve as a negative regulator of ABA response. Our study, described here, indeed confirmed this idea and identified by genetic analysis the first candidate of the AP2 transcription repressor

Table I. *Sequences of primers used for RT-PCR*

F, Forward primer; R, reverse primer.

Gene Name	Sequence (5′ to 3′)
RD29A	F: gaatggtgcgactaagatgtttaggaa
	R: gtacagattcagtgggtttggtgtaat
RD29B	F: gtgaagatgactatctcggtggtc
	R: gcctaactctccggtgtaacctag
RD22	F: gtggctaagaagaacgcaccgatgc
	R: ctctaattttattataggttttgcaaaac
RAB18	F: atgacgagtacggaaatccgatgg
	R: tatgtatacacgattgttcgaagc
COR47	F: atggctgaggagtacaagaacaacgtt
	R: tcttcttcttcttctccttcttttcct
COR15A	F: atggcgatgtctttctcaggagctgtt
	R: ttttatccgtcacgaaatctgaagctt

for ABA-regulated gene expression. A negative role for ABR1 in ABA inhibition of seed germination also seems to correlate with the functional role played by CBL9 and CIPK3 in this response. Based on the biochemical and genetic analyses of CBL9 and CIPK3, we speculate that they interact physically and functionally in the same ABA-signaling pathway (G.K. Pandey, Y. Cheong, and S. Luan, unpublished data). It is tempting to hypothesize that ABR1 protein may represent the TF that imposes a negative regulation to the ABA response downstream of CBL9-CIPK3. Although a direct interaction of ABR1 and CIPK3 was not identified in the yeast (*Saccharomyces cerevisiae*) two-hybrid system, it will be interesting to determine whether the ABR1 and the CBL-CIPK network are connected in any way in the regulation of ABA responses in plants.

MATERIALS AND METHODS

Plant Materials, Stress Treatments, and RNA Analysis

Arabidopsis (*Arabidopsis thaliana*) plants (ecotype Columbia) were grown in the greenhouse under long-day conditions (16-h-light/8-h-dark cycle) at 500 μmol m^{-2} s^{-1} and 21°C to 23°C, with 75% humidity for generation of seeds. For RNA analysis, 4-d-old seedlings grown on MS medium (Murashige and Skoog, 1962) were treated under different stress conditions. Seeds were treated with isopropanol for 5 min and with 50% bleach for 15 min, washed five times with sterile water, and plated on MS medium solidified with 0.9% agar.

For ABA treatment, 100 μM (±)-cis, trans-ABA solution in water was sprayed onto the 4-d-old seedlings grown on MS plates to ensure total coverage of the foliage area. Seedlings treated with ABA were incubated at room temperature under white light. In parallel experiments, water was sprayed as control. To perform drought treatments, 4-d-old seedlings grown in MS medium were exposed in the laminar flow hood for dehydration, as described previously (Kim et al., 2003). Total RNA was isolated with Tripure isolation reagent (Roche Diagnostics). All experiments were repeated at least three times, and results from one representative experiment are shown.

RT-PCR Analysis of Gene Expression

To examine the expression of *ABR1* and stress marker genes by RT-PCR, DNase I-treated, total RNA (2.5 μg) was denatured and subjected to RT reaction using Superscript II (200 units per reaction; Invitrogen) at 42°C for 50 min, followed by heat inactivation of the reverse transcriptase at 70°C for 15 min. PCR amplification was performed with initial denaturation at 94°C for 3 min, followed by 25 or 35 cycles of incubations at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min, using the full open reading frame of *ABR1* forward (5′-GAGGATCTAGAAGGAGGGTTTTGTATGTGT-3′) and reverse (5′-GAAGGGATCCATCAGGAGGATGGACTATTA-3′) primers to amplify a PCR product of 1.176 kb. Expression levels of *Actin2* were monitored with forward (5′-GGAAAGGATCTGTACGGTAAC-3′) and reverse (5′-TGTGAACGATTCCTGGAC-3′) primers to serve as a quantifying control. Similarly, semiquantitative RT-PCR reactions were performed by using the gene-specific primers for ABA and stress marker genes, such as *RD29A*, *RD29B*, *RD22*, *RAB18*, *COR47*, and *COR15A*. The primer sequences of the gene markers are listed in Table I. Aliquots of individual PCR products were resolved by agarose gel electrophoresis and visualized with ethidium bromide by Gel Doc 1000 (Bio-Rad).

Isolation of the *abr1* T-DNA Insertional Mutant Alleles

The *abr1* mutant alleles (*abr1-1* and *abr1-2*) were isolated from T-DNA insertional collections of Torrey Mesa Research Institute (*abr1-1* are SAIL140_F05) and SALK (http://signal.salk.edu; *abr1-2* are SALK_012151). The T-DNA borders of *abr1-1* and *abr1-2* alleles were defined by sequencing PCR products obtained using a T-DNA border primer SAILLB3 (5′-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3′) and SALKLB1 (5′-GCAAACCAGCGTGGACCGCTTGCTGCAACT-3′), respectively. The T-DNA insertion in the mutants (*abr1-1* and *abr1-2*) was confirmed by PCR and DNA gel-blot analysis, and its exact position was determined by sequencing. After selfing of heterozygous plants, homozygous *abr1* mutant alleles were identified by genomic and RT-PCR to confirm disruption of gene expression.

Germination and Root Elongation Assays

Approximately 100 seeds each from the wild-type, *abr1* mutant alleles (*abr1-1* and *abr1-2*) were planted in triplicate on MS agar medium with different concentrations of ABA, NaCl, mannitol, or Glc, and incubated at 4°C for 6 d before being placed at 23°C under long-day conditions. Germination (emergence of radicles) was scored daily for 9 d. The vertical germination and growth assays shown in Figure 3 were performed in a similar manner, except that the plates were placed vertically on a rack. Plant growth was monitored and photographed after 12 d. For root elongation assay, seeds were germinated and grown on MS agar medium for 3 d. Similar seedlings of wild type and mutants were transferred to MS agar containing different concentrations of ABA for 2 weeks before the root length was measured and recorded.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NM_125871.

Received May 31, 2005; revised July 30, 2005; accepted July 31, 2005; published October 14, 2005.

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